

Laser-Raman Investigation of Phospholipid-Polypeptide Interactions in Model Membranes[†]

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ABSTRACT: The interaction of aqueous dimyristoylphosphatidylcholine liposomes with the polypeptides gramicidin A, poly-L-lysine, valinomycin, and gramicidin S was investigated by means of laser-Raman spectroscopy. Auxiliary data were obtained with differential scanning calorimetry. Studies were carried out over the temperature range of 0–50 °C, encompassing the gel phase, the transition region, and the liquid crystalline phase of the liposomes. Conformational changes in the phospholipid molecules were investigated by

measuring the intensity of the 1062-cm⁻¹ Raman band which is assigned to C–C stretching vibrations of trans segments. Three different types of phospholipid-polypeptide interactions were indicated by the observed Raman data. They are interpreted as (a) orderly penetration of the phospholipid bilayer by a hydrophobic polypeptide; (b) polar interactions involving primarily the head groups of the phospholipid; and (c) disorderly hydrophobic binding between a polypeptide and the hydrocarbon domain of the phospholipid.

Raman spectroscopy has been shown to be an effective tool for structural studies of phospholipid molecules in natural as well as model membrane systems (Lippert & Peticolas, 1971; Larsson & Rand, 1973; Mendelsohn et al., 1975; Verma & Wallach, 1976; Yellin & Levin, 1977a–c). The sensitivity of several spectral features to conformational changes of the hydrocarbon chains provides a way to investigate the trans-gauche isomerism and, thus, the fluidity of bilayer assemblies (Lippert & Peticolas, 1971; Mendelsohn, 1972; Larsson & Rand, 1973; Gaber & Peticolas, 1977; Yellin & Levin, 1977a). 1,2-Diacylphosphatidylcholine–water bilayer systems are frequently accepted as models for the more complicated cellular membranes (Lippert & Peticolas, 1971; Mendelsohn, 1972; Mendelsohn et al., 1975; Gaber & Peticolas, 1977; Yellin & Levin, 1977c). The fluidity of model membranes in the gel

phase as a function of temperature has been investigated in some detail by Raman spectroscopy for systems composed of pure dimyristoyl, dipalmitoyl, and distearoyl phosphatidylcholine liposomes (Yellin & Levin, 1977a,b). Raman studies over a considerable temperature range have also been reported for the systems dipalmitoylphosphatidylcholine–cholesterol (Lippert & Peticolas, 1971) and dipalmitoylphosphatidylcholine–gramicidin A (Chapman et al., 1977; Weidekamm et al., 1977). Both cholesterol and gramicidin A increase the liposome fluidity in the gel phase and decrease it in the liquid crystalline phase.[°] Several Raman studies showing phospholipid-polypeptide interactions have been carried out at room temperature or at a few selected temperature values (Larsson & Rand, 1973; Verma & Wallach, 1976; Lis et al., 1976a,b). Related studies have been conducted with differential scanning calorimetry (Chapman et al., 1977; Papahadjopoulos et al., 1975; Chapman et al., 1974).

The present communication is concerned with a laser-Raman study of the interactions of several different polypeptides with the bilayers of 1,2-dimyristoyl-L-phosphatidylcholine as a function of temperature. Dimyristoyl-

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phosphatidylcholine was selected because its relatively low gel-to-liquid crystalline transition temperature facilitates the preparation of phospholipid-polypeptide complexes (Lis et al., 1976a). We are concerned with the manner in which the different polypeptides influence liposome structure above and below the main transition temperature and with the cooperativity of the transition.

Experimental Section

Materials and Methods. High-purity 1,2-dimyristoyl-L-phosphatidylcholine, gramicidin S, poly-L-lysine (molecular weight about 17 000), and valinomycin were obtained from Sigma Chemical Co.¹ Gramicidin A was purchased from Nutritional Biochemicals.

Pure phospholipid liposomes were prepared by weighing out 1,2-dimyristoyl-L-phosphatidylcholine and distilled water in a 1:4 ratio, mixing for 10 min on a vibrator type mixer, and equilibrating overnight at 50 °C. The systems containing poly-L-lysine and gramicidin S were prepared in the same way by weighing out and mixing the three components. The samples containing gramicidin A and those containing valinomycin were prepared by first dissolving the polypeptide and the phospholipid in benzene-methanol (95%:5% w/w). The solvent was blown off with dry nitrogen, and the remaining mixture was lyophilized overnight to remove traces of solvents. Liposomes were then prepared as previously described. The average sample size was ca. 0.1 g.

Laser-Raman spectra were obtained with a Spex Ramalog system equipped with an RCA C-31034 photomultiplier, photon counting, a Spectra-Physics Model 165-8 argon ion laser, and a Spex 180° viewing platform. A brass-jacketed cell was constructed for 180° excitation and connected with a thermostated water bath. The temperature was measured with a thermocouple located in the jacket close to the sample. The cell has the advantage over conventional capillaries and 90° scattering cells that only a shallow cavity has to be filled with the highly viscous suspension. The laser power was 400 mW at the sample, the spectral slit width was 5 cm⁻¹, and the 514.5-nm laser line was used for excitation. Local heating of the sample by the laser beam was estimated to be 2 °C at 30 and 39 °C, determined by comparison of the melting temperature of methyl palmitate and methyl stearate, both out of the beam and in the beam. The in-the-beam value was determined by observing the intensity change of the 1060-cm⁻¹ region Raman band as a function of temperature. All reported results take this correction into account.

Conformational changes in the phospholipid molecules were investigated by measuring the intensity of the 1062-cm⁻¹ Raman band, assigned to C-C stretching modes of trans segments of the hydrocarbon chains (Lippert & Peticolas, 1971; Spiker & Levin, 1975). The intensity of the C-N stretching mode at 715 cm⁻¹, which does not change with temperature (Gaber & Peticolas, 1977), was used as an internal reference. In principle, resolved band areas should be evaluated for intensity determinations. These are very difficult to determine in the crowded frequency region between ca. 1030 and 1130 cm⁻¹, where five bands occur, one of them the very broad and ill-defined ~1090-cm⁻¹ band assigned to gauche C-C stretching vibrations (Lippert & Peticolas, 1971; Spiker & Levin, 1975). We have therefore measured the peak intensities of two sharp bands, at 715 and 1062 cm⁻¹, with the tangent-line technique (Potts, 1963; Susi et al., 1964). The

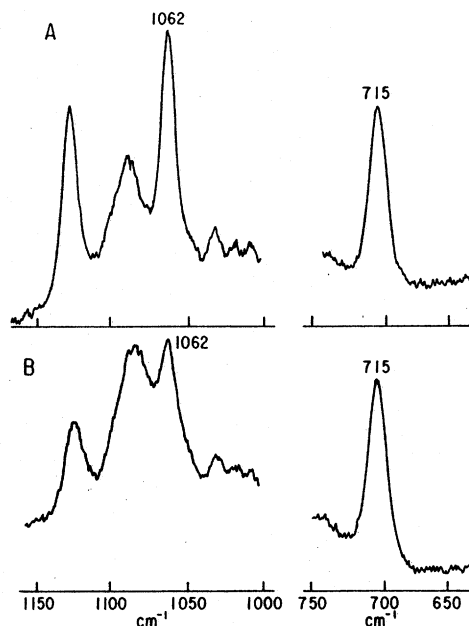


FIGURE 1: Representative sample of laser-Raman data. (A) The 1000–1150- and 650–750-cm⁻¹ spectral regions of the dimyristoyl-phosphatidylcholine-valinomycin complex at 0 °C; (B) the same at 20 °C. 10% w/w valinomycin. 1:4 w/w suspension in water.

Raman spectrum of a typical suspension of a phospholipid-polypeptide complex in the regions of interest to this study is shown at two different temperatures in Figure 1. The spectral contributions of the various polypeptides at frequencies close to the 1062-cm⁻¹ band at the 715-cm⁻¹ band were found to be negligible.

The differential scanning calorimetry measurements were carried out on a Perkin-Elmer Model DSC-1b instrument. Dispersions were pipetted into aluminum containers and sealed with a cold weld. Samples were weighed to ±0.005 mg on a Cahn Model RI electrobalance. The heating rate was 5 °C/min; the attenuation was usually 4×. The sample chamber was kept under dry nitrogen. Signal areas were evaluated with the help of an IBM 1130 digital computer.

Results and Discussion

Pure Phospholipid. In order to pinpoint phospholipid-polypeptide interactions, a relative intensity parameter (I_R) was defined at 1062 cm⁻¹ and evaluated for the pure phospholipid over the temperature range of 0–50 °C. The relative intensity (I_R) is defined analogously to the S parameter suggested by Gaber & Peticolas (1977), with the following changes. (1) We used the 1062-cm⁻¹ band instead of the 1133-cm⁻¹ band as a measure of trans content, because the frequency of the former is independent of temperature (Yellin & Levin, 1977c), and it is an almost pure trans C-C stretching mode (Schachtschneider & Snyder, 1963; Snyder, 1967). (2) We used the value obtained for the liposomes at liquid nitrogen temperature as the 100% trans standard (Yellin & Levin, 1977c) instead of the value for the solid phospholipid. I_R is thus defined as

$$I_R = (I_{1062}/I_{715}) / (I_{1062}/I_{715})_{\text{liquid nitrogen}}$$

The intensity values on the right-hand side of the equation are base-line intensities measured with the tangent-line technique. I_R has the value of one at liquid nitrogen temperature, where all C-C bonds are in the trans conformation and zero if none of them is in the trans conformation. Gaber & Peticolas (1977) further suggested that we can assume, as an approximation, that for an all-trans standard the intensity of the

¹ Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

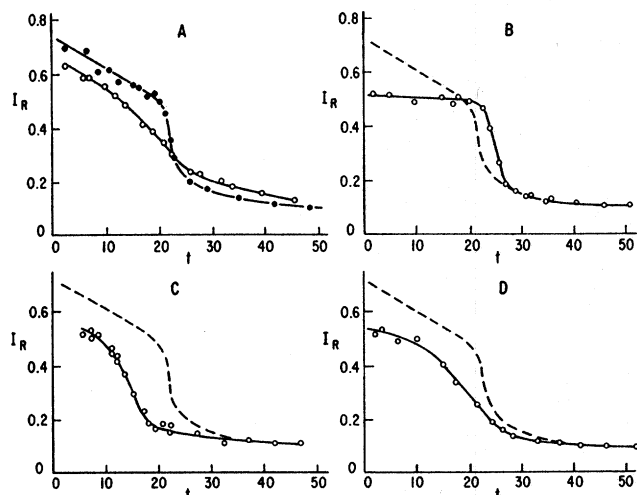


FIGURE 2: The relative intensity of the 1062-cm^{-1} trans band, I_R , as a function of temperature. $I_R = 1$ at liquid nitrogen temperature. (A) Pure dimyristoylphosphatidylcholine (DMPC) (\bullet — \bullet); DMPC plus 3% w/w gramicidin A (\circ — \circ). (B) DMPC plus 25% w/w poly-L-lysine. (C) DMPC plus 25% w/w gramicidin S. (D) DMPC plus 10% w/w valinomycin. All samples were prepared as 1:4 w/w suspensions in water. The dotted lines in B–D represent the I_R values of the pure phospholipid, for comparison.

trans bands is the sum of intensities associated with individual bonds. If we assume a similar additive relationship for shorter trans segments, the value of I_R becomes a measure for the fraction of C–C bonds in a trans conformation under a given set of conditions, i.e., an indicator of orderliness of the hydrocarbon chains.

Figure 2A gives I_R as a function of temperature for pure phospholipid liposomes (the line with filled circles). A slow, gradual decrease of orderliness is observed below the main transition temperature, as previously described for several different phosphatidylcholine liposomes (Yellin & Levin, 1977b,c). The main transition is reflected by a pronounced decrease of I_R .

Figure 3A₁ gives the heating curve obtained by differential scanning calorimetry. The calorimetric enthalpy change was found to be 5.4 ± 0.3 kcal/mol, in very good agreement with recent literature values (Mabrey & Sturtevant, 1976). We observe that the trans-gauche isomerization of the hydrocarbon chains as reflected in Raman spectra is a more gradual process than the calorimetrically observed phase transition.

Interaction with Gramicidin A. Gramicidin A is a linear pentadecapeptide which has been isolated from *Bacillus brevis* and has been frequently used as a model for an internal membrane protein which spans the bilayer (Chapman et al., 1977; Weidekamm et al., 1977). It is considered to form transmembrane channels through the association of two helical molecules via a hydrogen bond. The external hydrophobic residues are in contact with the hydrocarbon domain of the phospholipid, while the peptide carbonyl groups are thought to line a central hole axis of the helix (Urry, 1971; Veatch et al., 1974; Hladky & Haydon, 1972; Bamberg & Lauser, 1974; Veatch et al., 1975). It has been shown that gramicidin A increases the fluidity of dipalmitoylphosphatidylcholine liposomes in the gel phase and decreases the fluidity in the liquid crystalline phase, similarly to the behavior of cholesterol (Chapman et al., 1977; Weidekamm et al., 1977).

Figure 2A (open circles) gives the change of the orderliness of the hydrocarbon chains of dimyristoylphosphatidylcholine liposomes interacting with 3% (w/w) gramicidin A in terms of I_R as a function of temperature. There is no discernible sudden change at any temperature. Below the phase-transition

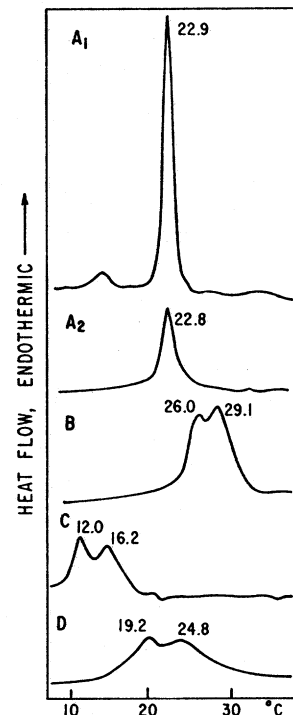


FIGURE 3: Differential scanning calorimetry heating curves. (A₁) Dimyristoylphosphatidylcholine (DMPC), 18×10^{-6} mol. (A₂) DMPC, 16×10^{-6} mol, plus 3% w/w gramicidin A. (B) DMPC, 23×10^{-6} mol, plus 25% w/w poly-L-lysine. (C) DMPC, 11×10^{-6} mol, plus 25% w/w gramicidin S. (D) DMPC, 26×10^{-6} mol, plus 10% w/w valinomycin. All preparations were 1:4 w/w suspensions in water.

temperature of pure phospholipid liposomes, the I_R value is about 10–20% lower than for the pure phospholipid liposomes; above this temperature, ca. 20–25% higher. A relatively small amount of a hydrophobic polypeptide which spans the phospholipid bilayer is thus capable of smoothing out the curve, eliminating any sudden “melting” of the hydrocarbon chains, as observed by Raman spectroscopy.

Figure 3A₂ shows the results obtained with differential scanning calorimetry. The main peak is considerably weaker than the one obtained with pure phospholipid liposomes, but the peak position and half-width are essentially unchanged; the pretransition peak has disappeared. The calculated ΔH value is 1.7 ± 0.3 kcal/mol, as compared to 5.4 kcal/mol for the pure phospholipid. Because the Raman data suggest a gradual change in the hydrocarbon chains, it appears that the sharp calorimetric peak could be caused primarily by a head group rearrangement.

Interaction with Poly-L-lysine. Poly-L-lysine has been used as a model for an extrinsic protein which interacts primarily with the polar end groups of phospholipid liposomes without penetrating extensively into the hydrocarbon domain (Papahadjopoulos et al., 1975; Hammes & Schullery, 1970). It has been concluded from results involving numerous physical techniques that the complex phosphatidyl-L-serine–poly-L-lysine, nevertheless, is stabilized by both electrostatic and hydrophobic interactions (Hammes & Schullery, 1970). With dipalmitoylphosphatidylglycerol, poly-L-lysine interacts in a manner which increases the transition temperature. This has been interpreted as a stabilization of the bilayer (Papahadjopoulos et al., 1975).

Figure 2B gives the change of I_R for dimyristoylphosphatidylcholine interacting with 25% (w/w) poly-L-lysine ($M_r \sim 1700$) in the temperature range of 0–50 °C. The curve for the pure phospholipid liposomes is given with a dotted line for comparison. Above ca. 25 °C both curves are identical;

i.e., the polypeptide has no effect on the hydrocarbon structure of the phospholipid. The transition midpoint is increased by ca. 4 °C and the transition is quite sharp, although a slight broadening has taken place. Below 20 °C the curve forms a plateau with a constant, relatively low I_R value. These data suggest that a certain stabilization of the trans structure has taken place as previously suggested (Papahadjopoulos et al., 1975), but it is limited to a narrow temperature range just above the transition temperature of pure phospholipid liposomes. Below the transition temperature the hydrocarbon chains appear to freeze into a rigid but relatively disordered structure. It is quite evident that poly-L-lysine does have an effect on the packing of the hydrocarbon chains in the gel phase, although the exact nature of this interaction is not obvious. Hydrophobic interactions appear to be one possibility (Hammes & Schullery, 1970).

Figure 3B gives the results obtained with differential scanning calorimetry. A doublet is observed, with both components above the transition temperature of pure phospholipid liposomes and the higher component considerably above the hydrocarbon transition of the same sample as observed by Raman spectroscopy. The combined ΔH value is 1.0 ± 0.2 kcal/mol. The higher component appears to be caused by a rearrangement at a temperature above the "melting" of the hydrocarbon chains as observed by Raman spectroscopy.

Interaction with Gramicidin S. Gramicidin S is a small, compact cyclic decapeptide antibiotic produced by a strain of *Bacillus brevis*. It contains five different kinds of hydrophobic amino acid residues, two of each. The molecule forms a rigid, β -type secondary structure with C_{2v} symmetry (Stern et al., 1968; Ohnishi & Urry, 1969). Most polar groups are tied up through hydrogen bonding in the skeletal backbone of the molecule. The hydrophobic groupings are outside on the periphery.

The temperature dependence of I_R for the phospholipid interacting with gramicidin S is shown in Figure 2C. We observe a substantial lowering of the transition temperature, a great broadening of the transition region (decrease of cooperativity), and a substantial decrease of order below the transition range. This behavior is quite different from the one observed for hydrophobic binding of gramicidin A (see above) and cholesterol, which are assumed to be built into the hydrocarbon domain of the phospholipid in an orderly manner, resulting in a stiffening of the bilayer in the liquid crystalline phase (Lippert & Peticolas, 1971; Chapman et al., 1977; Weidekamm et al., 1977). The relatively small and compact gramicidin S molecules are apparently embedded in the hydrophobic domain in a less orderly way. They decrease the cooperativity, the melting temperature of the hydrocarbon chains, and the orderliness in the gel phase but are not capable of inducing order into the liquid crystalline phase.

Figure 3C depicts the differential scanning calorimetry curve of the gramicidin S-phospholipid system. We observe a doublet with peaks at 12.0 and 16.2 °C, i.e., at substantially lower temperatures than the transition temperature for the pure phospholipid. The 12.0 °C peak coincides with the midpoint of the melting zone of the hydrocarbon chains, as estimated by Raman spectra (cf. Figure 2C); the 16.2 °C peak is above the center of the hydrocarbon melting region so determined. The combined calorimetric ΔH value is 1.1 ± 0.2 kcal/mol, as compared to 5.4 kcal/mol for the pure phospholipid.

Interaction with Valinomycin. Valinomycin is a cyclododecadesipeptide antibiotic produced by *Streptomyces*

fulvissimus. It increases alkali ion permeability of mitochondria (Pressman, 1968) and phospholipid liposomes (Johnson & Bangham, 1969) and affects the electrical properties of black lipid membranes (Krause et al., 1971). It has been suggested on the basis of a variety of physical and chemical studies that the molecule has surface-active properties and, on contact with phospholipid membranes, tends to accumulate mainly on their surface (Shemyakin et al., 1969). The uncomplexed molecule has a bracelet-like structure (Duax et al., 1972); the surface-active properties are supposedly caused by variously oriented carbonyls in the "upper" and "lower" parts of the molecule. On the other hand, the molecule is surrounded by hydrophobic groups in a manner somewhat similar to gramicidin S. Nuclear magnetic resonance studies have shown that valinomycin does penetrate the hydrophobic region of dimyristoylphosphatidylcholine and substantially affects the mobility of the terminal methyl groups of the hydrocarbon chains (Hsu & Chan, 1973).

Figure 2D shows the temperature dependence of I_R for dimyristoylphosphatidylcholine interacting with 10% (w/w) valinomycin. The curve is similar to the curve in Figure 2C which we ascribe to hydrophobic binding of gramicidin S to the hydrocarbon domain of the phospholipid; it is distinctly different from the curve in Figure 2B which results from polar binding to the head groups. We conclude that under the given conditions valinomycin penetrates into the hydrocarbon domain of the phospholipid but, like gramicidin S, does not induce order into the liquid crystalline phase, as observed with gramicidin A and cholesterol.

Figure 3D shows the differential scanning calorimetry results obtained with the phospholipid-valinomycin system. Curves 3C and 3D are qualitatively similar, but in 3D both peaks appear at higher temperatures. An examination of Figures 2C and 2D reveals that the spectroscopically determined transition region of the hydrocarbon chains of the valinomycin system also extends to considerably higher temperatures. The calorimetric peaks occur close to the midpoint of the spectroscopic transition curve and at a temperature corresponding to the onset of the liquid crystalline region. This is similar to the observations on the systems containing poly-L-lysine and gramicidin S and suggests that this higher peak is not directly related to hydrocarbon isomerism. The combined calorimetric enthalpy change is 4.0 ± 0.6 kcal/mol.

The four examined polypeptides thus interact with dimyristoylphosphatidylcholine in three distinctly different ways which can be characterized as orderly hydrophobic binding (gramicidin A), essentially polar binding (poly-L-lysine), and disorderly hydrophobic binding (gramicidin S and valinomycin). As compared to calorimetric measurements, laser-Raman spectroscopy offers two types of new information. It provides information about the nature of the system above and below the transition temperature and permits us to focus specifically on the structural changes taking place within the hydrocarbon domain of the bilayer.

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